

Oral supplementation of a cell preparation of *Enterococcus faecalis* strain EC-12 stimulates superoxide dismutase production in the livers of healthy and arthritis-induced mice

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Hepatitis, a major human chronic inflammation disease, has been linked to oxidative stress, which can be initiated by radicals produced during the oxidative metabolism. Oxidative damage has been also observed in arthritis-induced mice. Here we evaluated whether supplementation of a cell preparation of *Enterococcus faecalis* EC-12 could induce superoxide dismutase activity and/or damage in the livers of healthy mice or mice with arthritis. In Experiment 1, both healthy and arthritis-induced mice were orally given a saline solution, or a solution with a low (0.2 mg/mouse/day) or a high (2.0 mg/mouse/day) concentration of *E. faecalis* EC-12 for 49 consecutive days. Manganese superoxide dismutase activity increased in *E. faecalis* EC-12-supplemented mice but with no arthritis. In Experiment 2, mice received orally either a saline or an *E. faecalis* EC-12 suspension (10 mg/kg of body weight/day) for 28 consecutive days. No changes in tissues and levels of function markers and 8-hydroxy-2'-deoxyguanosine were observed in mouse livers, inferring that *E. faecalis* EC-12 supplementation caused no damage. While mRNA expression of copper/zinc superoxide dismutase remained unaltered, that of manganese superoxide dismutase increased in *E. faecalis* EC-12 administration mice. In conclusion, at least in healthy mice, *E. faecalis* EC-12 supplementation stimulated manganese superoxide dismutase activity in liver tissues with no side effects.

Key Words: *Enterococcus faecalis*, cell preparation, superoxide dismutase, liver, mouse

The development of hepatitis, one of the major chronic inflammation diseases in humans, has been linked to oxidative stress,⁽¹⁾ which is initiated by radicals produced during the oxidative metabolism.⁽²⁾ Indeed, while the oxidative metabolism occurs in all living organisms and is a crucial process for the survival of cells, it also produces nitrogen-based (e.g., nitric oxide radicals and peroxy-nitrite) and oxygen-based (e.g., hydroxyl radicals and superoxide) radicals.^(3,4) In the liver, which plays an important role in converting and excreting toxic substances⁽⁵⁾ and regulating metabolic homeostasis,⁽⁶⁾ free radicals likely cause damage to the cellular membrane of hepatocytes,⁽⁵⁾ peroxidation of lipids and breakage of DNA strands.⁽⁷⁾ Dysfunction of the liver caused by its damaged cells⁽⁸⁾ and hence its inability to dispose of toxic compounds⁽⁵⁾ then leads to occurrence of sustained inflammation,⁽⁹⁾ oxidative stress⁽¹⁰⁾ and in the end, diseases such as acute liver injury,⁽⁶⁾ hepatic encephalopathy, and non-alcoholic fatty liver.⁽⁵⁾

Rheumatoid arthritis (RA) is a systemic and chronic auto-

immune disease that leads to joint inflammation and progressive cartilage and bone erosion.^(11,12) Collagen-induced arthritis (CIA) is a T-cell dependent, induced to experimental animals suffering from RA. CIA-induced animals develop experimental arthritis with systemic inflammation after immunization with heterologous type II collagen.⁽¹³⁾ This chronic inflammation is usually associated with the development of oxidative stress,^(1,7) which was shown to cause systemic oxidative damage, particularly in liver and spleen CIA-induced mice.⁽¹⁴⁾

Probiotics is a general term to describe those, by definition,⁽¹⁵⁾ live bacterial strains that have been proven to confer health benefits to the host upon consumption of an adequate amount⁽¹⁶⁾ for a certain period of time. Nonetheless, at these premises we have demonstrated that supplementing certain lactic acid bacteria, regardless of their viability, to experimental models, induced a number of systemic responses. Those systemic responses ultimately resulted in improving the health of the experimental models⁽¹⁷⁻²⁰⁾ and ameliorating experimentally-induced disorders.^(21,22) Elsewhere, probiotic-supplemented rats also showed a low formation of lipid peroxides and a high anti-oxidant activity.⁽²³⁾ Separately, in a work in which *Bacillus* spp. was supplemented to rats, while it was observed a high activity of anti-oxidant enzymes catalase and superoxide dismutase (SOD) in liver tissues of the animals, none of them showed a deleterious effect of *Bacillus* spp. supplementation, neither due to a high dosage nor the length of the supplementation period.⁽²⁴⁾ Those results suggested that probiotics could be used as natural anti-oxidant supplements.⁽²⁵⁾

In the present study, we aimed to assess whether supplementation of a cell preparation of *Enterococcus faecalis* strain EC-12 (CPEF) could stimulate the activity of copper/zinc (Cu/Zn-SOD) and manganese (Mn-SOD) superoxide dismutases even if the bacterial strain was heat-killed. Due to SOD stimulation was needed to be characterized in mice suffering from a chronic inflammation condition and compare it with that of control mice, to increase the stimulation of hepatic SOD, in the present work we included not only healthy but also CIA-induced mice. As we theorized that a high SOD activity could well occur, we also aimed to assess if CPEF supplementation could cause inflammation and/or oxidative stress in the livers of mice. Therefore, we used 8-hydroxy-2'-deoxyguanosine (8-OH-dG) as the index of oxidative stress,⁽²⁶⁾ and enzymes aspartate (AST) and alanine

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(ALT) aminotransferases as markers to detect liver dysfunction and damage.⁽⁵⁾ To achieve the experimental objectives, two experiments were designed. In Experiment 1, we evaluated the possible hepatic SOD activity induced by CPEF supplementation in healthy and CIA-induced mice. To evaluate the stimulation of hepatic SOD activity, DBA/1J mice were used. In Experiment 2, we wanted to assess that no deleterious hepatic damage was caused by CPEF supplementation, and hence, healthy BALB/cCrSlc mice were used instead. To evaluate the hepatic stimulation of Cu/Zn-SOD and Mn-SOD after CPEF supplementation for 28 consecutive days, we also measured the mRNA levels in mice.

Materials and Methods

Bacteria preparation. The method for the cell preparation of CPEF was the same as that previously carried out at these premises.⁽²⁷⁾

Experiment 1. Experiment 1 was approved in accordance with the guidelines for animal studies of Kitasato University (Approval number SA0524). Ninety-two 8-week-old, male DBA/1Jcl mice, an arthritis model, were purchased from CLEA Japan (Tokyo, Japan) and introduced to a room at Kitasato University (Kanagawa, Japan). Mice were kept in an air-conditioned room (temperature, $24 \pm 2^\circ\text{C}$; humidity $50 \pm 5\%$) in a 12-h light (08:00 am–20:00)-dark cycle. All mice received an MF chow diet (Oriental Yeast Co. Ltd., Tokyo, Japan) and water *ad libitum*. After 7-day acclimatization, mice with comparable body weight were first divided into 2 groups: non-arthritic and arthritic mice. These groups were further divided into 3 subgroups each: groups given a saline solution (non-arthritic control, group nC; $n = 10$), a low (non-arthritic-CPEF 0.2 mg/mouse/day, group nL; $n = 10$) or a high concentration of CPEF (non-arthritic-CPEF 2.0 mg/mouse/day, group nH; $n = 10$). The other 3 subgroups were given the saline solution (arthritic-control, group iC; $n = 23$), a low (arthritic-CPEF 0.2 mg/mouse/day, group iL; $n = 19$) or a high concentration of CPEF (arthritic-CPEF 2.0 mg/mouse/day, group iH; $n = 20$). All mouse groups were housed in plastic cages throughout the study.

CPEF was suspended in sterile saline and given orally to mice with a commercial, disposable feeding needle (Fuchigami, Kyoto, Japan), every day at 10:00 am for 49 consecutive days (day 0–48).

CIA-induced mice (iC, iL, and iH) were subcutaneously injected 0.1 ml of a suspension of type II collagen (originated from chick sternal cartilage; Sigma Aldrich Japan, Tokyo, Japan) at the base of the tail on days 0 and 21. The collagen suspension was prepared as follows. Type II collagen was dissolved in 10 mmol/L of acetate (2 mg/ml), and then emulsified (1:1) with Freund's complete adjuvant. Freund's complete adjuvant was prepared by adding heat-killed *Mycobacterium tuberculosis* strain H37Ra into Freund's incomplete adjuvant (Becton, Dickinson and Co., Sparks, MD) at a concentration of 5 mg/ml. All mice injected with the collagen suspension positively developed CIA from day 24. Clinical observation of arthritis in each foot was checked thrice per week from days 24 to 49.

At the end of the experiment (day 49), all mice underwent anesthesia with isoflurane via inhalation (MSD, Tokyo, Japan) and dissected while being under deep anesthesia. After exsanguination, the entire livers of mice were immediately removed and quickly stored at -80°C until analysis. SOD activity was measured in the entire livers of mice with a commercial assay kit (SOD Assay Kit – WST; Dojindo Laboratories, Kumamoto, Japan). The concentration of protein in the liver was also measured with a commercial kit (Proteostain Protein Quantification Kit Rapid; Dojindo Laboratories). The assays were conducted as per the manufacturer's instructions. Cu/Zn-SOD activity was calculated as: Cu/Zn-SOD activity = total

SOD activity – Mn-SOD activity.

Experiment 2. Experiment 2 was approved in accordance with the guidelines for animal studies of the Kyoto Institute of Nutrition & Pathology (Kyoto, Japan; Approval number 07047CM). Fifteen 8-week-old, male BALB/cCrSlc mice were purchased from SLC Japan (Shizuoka, Japan). Mice were kept in an air-conditioned room (temperature, $24 \pm 2^\circ\text{C}$; humidity $45 \pm 10\%$) in a 12-h light (08:00 am–20:00)-dark cycle at the Kyoto Institute of Nutrition & Pathology. All mice received an MF chow diet (Oriental Yeast Co. Ltd., Tokyo, Japan) and water *ad libitum*. After 7-day acclimatization to the experimental settings, mice with comparable body weight were divided into 2 groups and given either a saline solution (control, group C; $n = 7$) or CPEF (10 mg/kg of body weight; group T; $n = 8$). The amount of supplementation for group T was approximately the same as that for groups nL and iL in Experiment 1. Mouse groups were housed in plastic cages throughout the study.

CPEF was suspended in sterile saline and given orally to mice with a commercial, disposable feeding needle (Fuchigami), every day at 10:00 am for 28 consecutive days (day 0–27).

At the end of the experiment (day 28), all mice were administered an intraperitoneal injection of sodium pentobarbital (Somnopentyl; Kyoritsu, Tokyo, Japan) and dissected under deep anesthesia. Before exsanguination, blood was collected from the abdominal vein. After exsanguination, the right medial lobes of the livers of mice were removed, immediately soaked into RNA-later solution (Sigma-Aldrich Japan, Tokyo, Japan), and maintained overnight at 4°C , as per the manufacturer's instructions. Liver samples were then stored at -80°C until further analysis. Separately, the left lateral lobes of the livers were fixed using a 10% neutralized formalin solution. The remaining of the livers was stored at -80°C until further analysis.

The levels of AST and ALT in the serum of mice were measured by Japan Clinical Laboratory (Kyoto, Japan) using a consensual method.⁽²⁸⁾ The concentration of cellular oxidative stress marker 8-hydroxy-2'-deoxyguanosine per 10^6 DNA bases (8-OH-dG/ 10^6 dG) was measured by the OHG Institute Co., Ltd. (Fukuoka, Japan). The analysis procedure was the same as that previously described.⁽²⁶⁾ Fixed liver tissues were embedded into paraffin wax and cut into 3- μm -thick cross-sections. Cross-sections were then prepared and stained with hematoxylin and eosin. A veterinarian at Kyoto Institute of Nutrition & Pathology with histopathology expertise screened the samples for histopathological abnormalities using a light microscopy (BX-51; Olympus, Tokyo, Japan) in a blind manner. The methods used for total RNA extraction, cDNA synthesis, and real-time polymerase chain reaction (PCR) have been described elsewhere.⁽²⁹⁾ Primers and TaqMan probes used in the present study are listed in Table 1.

Statistical analysis. First, we conducted a complete randomized design 2-way ANOVA (factors: arthritis and CPEF supplementation) to analyze the differences between the means of the experimental parameters in Experiment 1. Second, for all parameters for which an interaction effect ($p < 0.05$) was detected, a complete randomized design 1-way ANOVA of all 6 groups (groups nC, nL, nH, iC, iL, and iH) was applied. When a parameter in a given experimental group was different, the Tukey–Kramer post-hoc was used instead to compare it with those in other groups and detect statistical significances.

Depending on the results of the F-test, the Student's *t* test was or was not used to analyze the differences between the means of samples in Experiment 2.

Data are shown as the means \pm SE. In all statistical analyses, the differences between the means were considered significant at $p < 0.05$. All data were analyzed with Statcel3 (OMS, Saitama, Japan), an add-in application for Microsoft Excel (Microsoft, Seattle, WA).

Table 1. Primers and probes used in the present study

Gene name	Primers 5'-3'	GenBank accession number
Copper/zinc superoxide dismutase (Cu/Zn-SOD) (<i>sod1</i>)	F ctctcaggagagcattccatcat R caggaatgtttactctgcga P ccgtacaatgggtggtccatgagaacaa	NM_011434.1
Manganese superoxide dismutase (Mn-SOD) (<i>sod2</i>)	F gaacttcagtgcaggctgaaga R aacgccaccgaggagaagt P tgtaacatctcccttggccagagcctc	NM_013671.3
Glyceraldehyde 3-phosphate dehydrogenase (<i>gapdh</i>)	F ggtgtcttcaccacatgga R cagaaggggcccagatgat P aagggccgggcccacttgaa	NM_008084.2

Primers and probes were designed and synthesized by Bioresearch Technologies Japan (Tokyo, Japan).

Results

Experiment 1. Typical arthritis was observed in iC, iL, and iH groups during this experiment. CIA was gradually severe from days 24 to 35, whereas CIA was still observed at days 49. On the other hands, remarkable difference of clinical observations was not observed among the groups (data not shown).

Hepatic SOD activity (Cu/Zn-, Mn-, and total-SOD) was either highly ($p < 0.01$) detected in non-arthritis mice but not in CIA-induced mice (factor arthritis; Fig. 1). Indeed, when the activity of Cu/Zn-SOD was assessed, while no significant differences were detected between group nC and group nH, the activity of this enzyme in group nL tended to decrease, although this decrease was not statistically significant (Fig. 1A). Moreover, Mn-SOD activity was detected to be higher in groups nL and nH (non-arthritis CPEF supplementation mice) than in the other mouse groups (Fig. 1B). However, no statistical significance was detected between iC, iL, and iH groups. Total SOD activity increased in a dose-dependent manner by CPEF in CIA-induced mice, but no statistical significance was detected between iC, iL, and iH groups. (Fig. 1C).

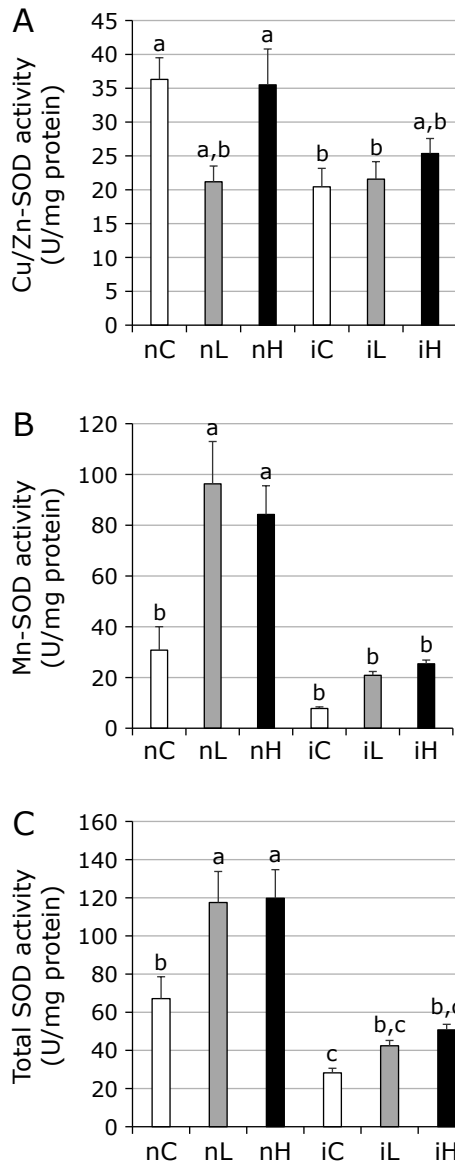
Experiment 2. Commonly recognizable histopathological abnormalities such as hepatocyte necrosis, granulocyte infiltration, lymphocyte infiltration, macrophage exudation and capsule hyperplasia were undetected in the livers of mice (data not shown). With respect to the gene expression of SODs in the liver, Mn-SOD was higher ($p < 0.05$) in CPEF-supplemented than in unsupplemented mice. By contrast, no significant differences in Cu/Zn-SOD were detected between mouse groups (Fig. 2A). This mRNA expression was very similar with that of SOD activity in Experiment 1, therefore we concluded that CPEF also stimulated SOD activity in the liver of BALB/c mice in Experiment 2. Apart from that, no significant differences in serum levels of AST and ALT were found between unsupplemented or CPEF-supplemented mice (Fig. 2B). Similarly, no significant differences were found in the concentrations of 8-OH-dG/ 10^6 dG in the livers of unsupplemented and CPEF-supplemented mice (Fig. 2C).

Discussion

A surge in SOD activity *in vivo* is usually an indication of high anti-oxidant activity.^(30,31) In this context, we observed that in Experiment 1, no significant differences in the Cu/Zn-SOD activity between untreated and CPEF-treated mice were detected regardless of whether mice was suffering from CIA or not (Fig. 1A). In contrast, using Mn-SOD as the marker, it was detected that SOD activity was higher ($p < 0.05$) in the liver tissues of CPEF-treated mice than in untreated mice in non-arthritis mice (Fig. 1B). While Cu/Zn-SOD can be found mainly in the eukaryotic cytoplasm,^(32,33) Mn-SOD usually exists in the

mitochondrion, which is especially vulnerable to oxidation due to over 90% of the cell's oxygen is spent within this organelle.⁽³⁴⁾ However, Mn-SOD is also produced in many Gram-positive bacteria as a protective mechanism against bactericidal action,⁽³⁵⁾ such as exogenous stress.^(31,36) Since *E. faecalis* is also a Gram-positive bacterium, the present data seem to confirm that the increased gene expression of Mn-SOD observed in the livers of mice was induced by supplementation of CPEF, but not by a bacterial activity of *E. faecalis*, because CPEF cells were heat-killed.⁽²⁷⁾ Ayyanna *et al.*⁽²³⁾ reported that SOD activity was associated with anti-inflammatory cytokines such as IL-10, the production of which was stimulated by ingestion of probiotics. As CPEF supplementation has been previously shown to stimulate production of IL-10 in human peripheral blood mononuclear cells,⁽³⁷⁾ it is likely that in the present work, supplementation of CPEF also activated SOD, which resulted in increased IL-10 production.

Qian *et al.*⁽³⁸⁾ and Suo *et al.*⁽³⁹⁾ reported that *L. plantarum* CQPC11 and *L. fermentum* Suo upregulated both Cu/Zn-SOD and Mn-SOD in liver tissues of mice undergoing D-galactose-induced oxidation and in a mouse model of gastric injury, respectively. However, in Experiment 1 of the present study, while the total SOD activity (Cu/Zn-SOD + Mn-SOD) was equally higher in the treatment groups when compared with control mice (Fig. 1C), when looking at the individual SOD activity, the Mn-SOD activity was higher in the livers of mice receiving both low and high concentrations of CPEF in healthy condition (Fig. 1B). However, with respect to Cu/Zn-SOD, not only did it not increase in the livers of mice, but it actually decreased in those receiving a low concentration of CPEF (Fig. 1A). Only a handful of animal studies have showed SOD data as separate compounds (Cu/Zn-SOD and Mn-SOD); the majority has reported it as the total SOD.^(31–33,39,40) Therefore, of interest here, it is the discrepancy between our data and those from studies reporting separate measurements of both Cu/Zn-SOD and Mn-SOD expression. A possible interpretation of our results would lie in the fact that in the end, CPEF administration did not cause evident hepatic dysfunction and damages on mice post-ingestion, which would also explain the lack of significant differences in the levels of AST, ALT, and 8-OH-dG/ 10^6 dG (Figs. 2B and C) between untreated or CPEF-treated mice. The fact that no physical abnormalities were observed in fixed liver tissues of mice in Experiment 2, further supports our assumption. Taking into consideration all this evidence, the decrease of Cu/Zn-SOD in livers of mice receiving a low concentration of CPEF would explain itself, as even a high concentration of CPEF would not induce an increase in the expression of Cu/Zn-SOD. Moreover, it is likely that the high activation of Mn-SOD observed in treatments groups was similar to that observed in Experiment 1, that is to say, resulting from metabolic processes in hepatic mitochondria, due to CPEF administration. It can be then inferred that



2-way ANOVA *p* value

Factors	<i>p</i> value
Arthritis	0.001
CPEF	0.06
Interaction	0.04

2-way ANOVA *p* value

Factors	<i>p</i> value
Arthritis	<0.001
CPEF	<0.001
Interaction	<0.001

2-way ANOVA *p* value

Factors	<i>p</i> value
Arthritis	<0.001
CPEF	<0.001
Interaction	0.04

Fig. 1. Superoxide dismutase activity in the livers of healthy or arthritis-induced mice and untreated or treated with *Enterococcus faecalis* strain EC-12 in Experiment 1. (A) copper/zinc superoxide dismutase (Cu/Zn-SOD) activity. (B) manganese superoxide dismutase (Mn-SOD) activity. (C) total SOD activity. Cu/Zn-SOD activity was determined by subtracting the total SOD activity from the Mn-SOD activity, as per the instructions of the material's manufacturers. Bars with different superscripts indicate data differ significantly ($p < 0.05$). White bars: mice given saline solution [non-arthritis mice (nC) and arthritis mice (iC); $n = 10$ and $n = 23$, respectively]. Gray bars: mice administered with a low concentration of CPEF [0.2 mg/mouse/day; non-arthritis mice (nL) and arthritis mice (iL); $n = 10$ and $n = 19$, respectively]. Black bars: mice administered a high concentration of CPEF [2.0 mg/mouse/day; non-arthritis mice (nH) and arthritis mice (iH); $n = 10$ and $n = 20$, respectively].

consecutive CPEF supplementation of 0.2 mg/mouse/day was enough to stimulate the SOD activity in the liver of mouse at least in healthy condition, because total SOD activity levels were almost the same in groups nL and nH in Experiment 1. It is worth noting that modulation of the intestinal microbiota is closely associated with anti-oxidant activity.⁽³⁷⁾ Since not only live but also non-viable lactic acid bacteria have been shown to modulate the intestinal microbiota,⁽¹⁸⁾ we theorize that CPEF could also potentially be a microbiota modulator in the gut,⁽⁴¹⁾ which in turn may be associated with SOD activity in the liver. We suggest that this scenario be further investigated in the future.

Artificially-induced arthritis typically caused a reduction of SOD activity in the livers of mice (Fig. 1). This result was similar to those reported in other murine arthritis models.^(14,42) Although previous studies reported that anti-oxidant administra-

tion suppressed the oxidative stress in CIA-induced mice,⁽⁴³⁾ in the present study, SOD production in the livers of CIA-induced mice was not stimulated by CPEF supplementation (Fig. 1). In contrast, CPEF supplementation either greatly induced the production of Mn-SOD or total SOD in the livers of non-arthritis mice. Since previous work suggested that SOD reduction was more often detected in the spleen than in the liver,^(14,42) it could be theorized that by using spleen samples, we could have detected changes in SOD activity. Nonetheless, as the use of spleen samples was beyond the scope of the present work, it is strongly suggested that spleen samples be screened for SOD activity in future research.

In conclusion, in the present study we showed that, at least in healthy mice, heat-killed CPEF stimulated the total SOD activity in liver tissues. Oxidative stress caused by CIA significantly

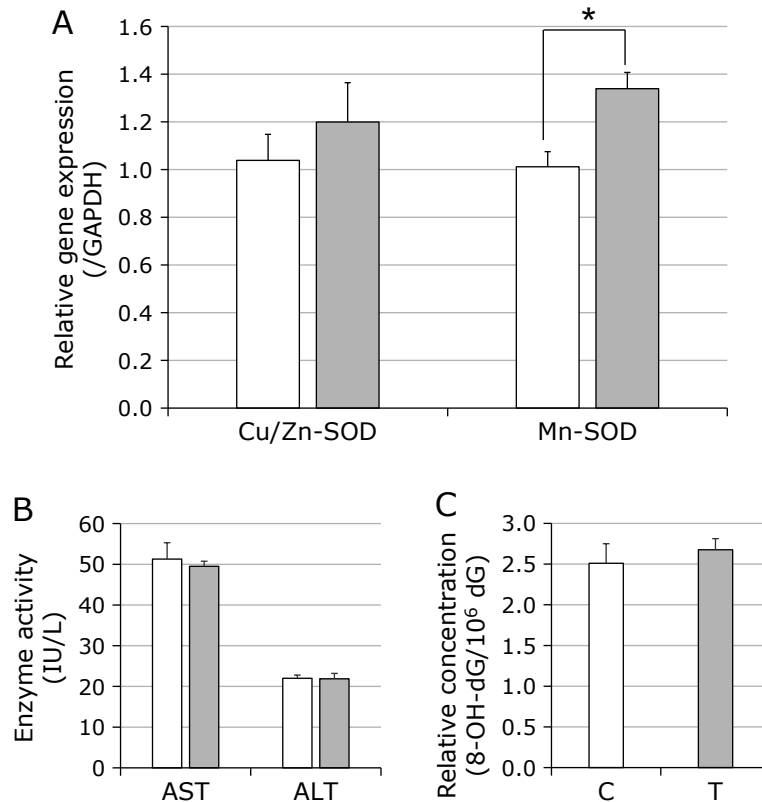


Fig. 2. Analysis of superoxide dismutase expression and functional damage in the livers of untreated and CPEF-treated mice in Experiment 2. (A) mRNA expressions of copper/zinc superoxide dismutase (Cu/Zn-SOD) and manganese superoxide dismutase (Mn-SOD). (B) activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in the serum of mice. (C) the term 8-OH-dG/10⁶ dG refers to the relative concentration of 8-hydroxy-2'-deoxyguanosine per 10⁶ DNA bases, in the livers of mice. The asterisk indicates significant differences ($p < 0.05$) between mouse groups. White bars: mice given a saline solution (group C; $n = 7$). Gray bars: mice given CPEF (10 mg/kg of body weight/day; group T; $n = 8$). The amount of CPEF administration for group T was approximately the same as that for groups nL and iL in Experiment 1.

reduced SOD activity, but CPEF supplementation had only a limited effect on SOD production. Finally, no deleterious effect of CPEF supplementation on mice was observed, as confirmed by the visual inspection of the liver tissues of mice and the measurement of circulating AST, ALT, and 8-OH-dG/10⁶ dG levels.

Author Contributions

YM-I, TI, and HT designed the experiments. YM-I and TI supplied the materials. HT and TT conducted the experiments. TT carried out the statistical analysis and wrote the draft of the manuscript. TI was responsible for the overall direction of the project and for editing the manuscript. All authors contributed to the article and approved the submitted version.

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dG/10⁶ dG. We also thank Bioscience Proofreaders (<https://bioscience-proofreaders.com>) for editing the English language of this manuscript.

Abbreviations

ALT	alanine aminotransferase
AST	aspartate aminotransferase
CIA	collagen-induced arthritis
CPEF	cell preparation of <i>Enterococcus faecalis</i> strain EC-12
Cu/Zn-SOD	copper/zinc superoxide dismutase
Mn-SOD	manganese superoxide dismutase
8-OH-dG	8-hydroxy-2'-deoxyguanosine
PCR	polymerase chain reaction
RA	rheumatoid arthritis
SOD	superoxide dismutase

Conflict of Interest

YM-I and TI are employed by Combi Corporation. TT is employed by Kyoto Institute of Nutrition & Pathology, which has received research funding from Combi Corporation. HT had no conflict of interest.

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